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(12) (19) (CA) **Demande-Application**

(21) (A1) **2,255,284**
(22) 1998/12/17
(43) 1999/06/22

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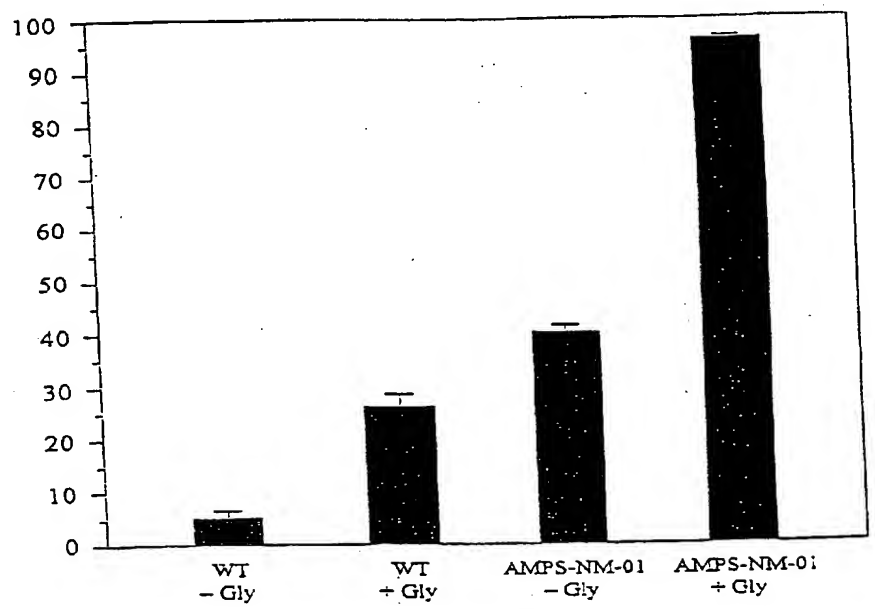
(51) Int.Cl.[6] C12N 15/54, C12N 15/80, C12N 1/15, C12N 1/14, C12P 25/00
(30) 1997/12/22 (197 57 180.8) DE
(30) 1998/09/09 (198 40 709.2) DE

(54) **ORGANISMES UNICELLULAIRES OU PLURICELLULAIRES UTILISES DANS LA PREPARATION DE RIBOFLAVINE**

(54) **UNICELLULAR OR MULTICELLULAR ORGANISMS FOR PREPARING RIBOFLAVIN**

Drawings



(57) The present invention relates to a unicellular or multicellular organism, in particular a micro-organism, for biotechnologically preparing riboflavin. This organism is distinguished by the fact that it exhibits a glycine metabolism which is altered such that its synthetic output of riboflavin without any external supply of glycine is at least equal to that of a wild type of the species Ashbya gossypii, i.e. ATCC10895, which is cultured under standard conditions with the addition of 6 g of external glycine/l.

Abstract of the disclosure

The present invention relates to a unicellular or multicellular organism, in particular a micro-organism, for biotechnologically preparing riboflavin. This organism is distinguished by the fact that it exhibits a glycine metabolism which is altered such that its synthetic output of riboflavin without any external supply of glycine is at least equal to that of a wild type of the species Ashbya gossypii, i.e. ATCC10895, which is cultured under standard conditions with the addition of 6 g of external glycine/l.

Unicellular or multicellular organisms for preparing riboflavin

The present invention relates to a unicellular or multicellular organism for preparing riboflavin using microorganisms.

Vitamin B_2, also termed riboflavin, is essential for humans and animals. Inflammations of the oral and pharyngeal mucous membranes, cracks in the corners of the mouth and pruritus and inflammations in the skin folds, among other damage to the skin, conjunctival inflammations, diminished visual acuity and clouding of the cornea appear in association with vitamin B_2 deficiency. Cessation of growth and decrease in weight can occur in infants and children. Vitamin B_2 therefore is of importance economically, in particular as a vitamin preparation in association with vitamin deficiency and as a feed additive. In addition to this, it is also employed as a foodstuff colorant, for example in mayonnaise, ice cream, blancmange, etc.

Riboflavin is prepared either chemically or microbially. In the chemical methods of preparation, the riboflavin is as a rule isolated in multi-step processes as a pure end product, with, however, relatively expensive starting compounds, such as D-ribose, having to be employed. For this reason, the chemical synthesis of riboflavin is only suitable for those applications for which pure riboflavin is required, for example in human medicine.

Using microorganisms to prepare riboflavin offers an alternative to preparing this substance chemically. Preparing riboflavin microbially is particularly suitable in those instances in which this substance is not required to be of high purity. This is the case, for example, when the riboflavin is to be employed as an additive to feed products. In such

cases, the microbial preparation of riboflavin has the advantage that the riboflavin can be obtained in a one-step process. In addition, renewable raw materials, such as vegetable oils, can be employed as starting compounds for the microbial synthesis.

It is known to prepare riboflavin by fermenting fungi such as Ashbya gossypii or Eremothecium ashbyi (The Merck Index, Windholz et al., eds. Merck & Co.), page 1183, 1983, A. Bacher, F. Lingens, Augen. Chem. 1969, p. 393); however, yeasts, such as Candida or Saccharomyces, and bacteria, such as Clostridium, are also suitable for producing riboflavin.

Methods using the yeast Candida famata are also described, for example in US 05231007.

Bacterial strains which overproduce riboflavin are described, for example, in EP 405370, GB 1434299, DE 3420310 and EP 0821063, where the strains were obtained by transforming the riboflavin biosynthesis genes from Bacillus subtilis. However, these prokaryotic genes were unsuitable for a method of preparing riboflavin recombinantly which used eukaryotes such as Saccharomyces cerevisiae or Ashbya gossypii. For this reason, the specific genes for riboflavin biosynthesis were, as described in WO 93/03183, isolated from a eukaryote, namely from Saccharomyces cerevisiae, in order thereby to provide a recombinant method for preparing riboflavin in a eukaryotic production organism. However, recombinant preparation methods of this nature are either unsuccessful, or only enjoy limited success, in producing riboflavin if there is inadequate provision of substrate for the enzymes which are specifically involved in the riboflavin biosynthesis.

In 1967, Hanson (Hanson AM, 1967, in Microbial Technology, Peppler, HJ, pp. 222-250, New York) found that adding the amino acid glycine increases the formation of riboflavin in Ashbya gossypii. However, such a method is disadvantageous because glycine is a

very expensive raw material. For this reason, efforts were made to optimize riboflavin production by preparing mutants.

German Patent Specification 19525281 discloses a method for preparing riboflavin which involves culturing microorganisms which are resistant to substances which have an inhibitory effect on isocitrate lyase.

German Laid-Open Specification 19545468.5-41 discloses another method for preparing riboflavin microbially in which the isocitrate lyase activity or the expression of the isocitrate lyase gene of a riboflavin-producing microorganism is increased. However, even in comparison with these methods, there is still a need for a further optimization of the riboflavin preparation.

The object of the present invention is consequently that of making available a unicellular or multicellular organism, preferably a microorganism, for the biotechnological preparation of riboflavin, which microorganism enables formation of the riboflavin to be further optimized. In particular, an organism should be made available which is suitable for preparing riboflavin while economizing on raw materials and which consequently makes possible a production which is more economical than that of the previous state of the art. In particular, the organism should permit an increased formation of riboflavin, without any addition of glycine, as compared with the previous organisms.

This object is achieved by means of a unicellular or multicellular organism which exhibits a glycine metabolism which is altered such that its synthetic output of riboflavin without any external supply of glycine is at least equal to that of a wild type of the Ashbya gossypii species ATCC10895, which is cultured under standard conditions with the addition of 6 g of external glycine/l.

Culturing under standard conditions means culturing, at 30°C and 120 rpm, in 500 ml shaker flasks possessing two baffles. 50 ml of a solution of 10 g of yeast extract/l containing either 10 g of glucose/l or 10 g of soybean oil/l are employed per flask as the medium. The media are inoculated with 1% of a 16 h culture carried out under the same conditions.

The objective of this sought-after alteration of the intracellular metabolism of glycine can be achieved using the known methods for improving organism strains. This means that, in the simplest case, appropriate strains can be prepared by means of screening after the selection which is customary in microbiology. It is also possible to use mutation in conjunction with subsequent selection. In this case, the mutation can be carried out either by means of chemical mutagenesis or by means of physical mutagenesis. A further method is that of selection and mutation together with subsequent recombination. Finally, the organisms according to the invention can be prepared by means of genetic manipulation.

According to the invention, the organism is altered such that it produces glycine intracellularly in a quantity which is greater than its requirement for maintaining its metabolism. According to the invention, this increase in intracellular glycine production can be achieved by preparing an organism in which the activity of the enzyme threonine aldolase is increased. This can be achieved, for example, by increasing substrate turnover by means of altering the catalytic center or by abolishing the effect of enzyme inhibitors. An increase in the activity of the threonine aldolase enzyme can also be elicited by increasing the synthesis of the enzyme, for example by means of gene amplification or by eliminating factors which repress the biosynthesis of the enzyme.

According to the invention, the endogenous threonine aldolase activity can preferably be increased

by mutating the endogenous threonine aldolase gene. Such mutations can either be produced randomly by means of classical methods, such as using UV irradiation or mutation-provoking chemicals, or in a targeted manner using genetic engineering methods such as deletion, insertion and/or nucleotide exchange.

Increased expression of the threonine aldolase gene can be achieved by incorporating copies of the threonine aldolase gene and/or by enhancing regulatory factors which exert a positive effect on threonine aldolase gene expression. For example, regulatory elements can preferentially be enhanced at the transcriptional level by, in particular, increasing the transcription signals. In addition to this, however, it is also possible to enhance translation by, for example, improving the stability of the mRNA.

In order to increase the gene copy number, the threonine aldolase gene can, for example, be incorporated into a gene construct or a vector which preferably contains regulatory gene sequences which are assigned to the threonine aldolase gene, in particular those sequences which enhance gene expression. A riboflavin-producing microorganism is then transformed with the gene construct containing the threonine aldolase gene.

According to the invention, the threonine aldolase can also be overexpressed by exchanging the promoter. In this context, it is also possible to achieve the higher enzymic activity in an alternative manner by incorporating gene copies or by exchanging the promoter. However, it is equally also possible to achieve the desired alteration in the enzymic activity by simultaneously exchanging the promoter and incorporating gene copies.

Since threonine is limiting in an organism which has been altered in this way, it is necessary to feed in threonine when the cell according to the invention is employed. The improved uptake of the

threonine and its virtually quantitative conversion into glycine lead to a surprisingly large increase in riboflavin formation such as was not previously achievable by feeding in glycine. Alternatively, the endogenous formation of threonine in the organism can be increased, for example, by eliminating the feedback resistance of the aspartate kinase.

The threonine aldolase gene is preferably isolated from microorganisms, particularly preferably from fungi. Fungi of the genus Ashbya are once again preferred in this context. The species Ashbya gossypii is highly preferred.

However, all other organisms whose cells contain the sequence for forming threonine aldolase, that is animal and plant cells as well, are also suitable for isolating the gene. The gene can be isolated by means of homologous or heterologous complementation of a mutant which is defective in the threonine aldolase gene or by means of heterologous probing or PCR using heterologous primers. For subcloning, the size of the insert in the complementing plasmid can subsequently be reduced to a minimum by means of suitable restriction enzyme steps. After the putative gene has been sequenced and identified, subcloning which gives an accurate fit is effected by means of fusion PCR. Plasmids which carry the resulting fragments as inserts are introduced into the threonine aldolase gene-defective mutant, which is then tested for the functionality of the threonine aldolase gene. Functional constructs are finally used to transform a riboflavin producer.

Following isolation and sequencing, the threonine aldolase genes can be obtained with nucleotide sequences which encode the given amino acid sequence or its allelic variation. Allelic variations include, in particular, derivatives which can be obtained by deleting, inserting or substituting nucleotides from appropriate sequences while at the

same time retaining the threonine aldolase activity. A corresponding sequence, from nucleotide 1 to nucleotide 1149, is shown in Figure 2b.

A promoter having the nucleotide sequence from nucleotide -1231 to nucleotide -1 as depicted in the abovementioned sequence, or a DNA sequence which has essentially the same effect, is, in particular, placed upstream of the threonine aldolase genes. Thus, a promoter which differs by one or more nucleotide substitutions, by insertion and/or by deletion from the promoter which possesses the nucleotide sequence shown without, however, the functionality or the activity of the promoter being impaired, can, for example, be placed upstream of the gene. In addition, the activity of the promoter can be increased by altering its sequence, or the promoter can be completely replaced by active promoters.

Moreover, regulatory gene sequences or regulatory genes which, in particular, increase the activity of the threonine aldolase gene can be assigned to the threonine aldolase gene. Thus, enhancers, which increase threonine aldolase gene expression by improving the interaction between the RNA polymerase and the DNA, can, for example, be assigned to the threonine aldolase gene.

One or more DNA sequences can be placed upstream and/or downstream of the threonine aldolase gene, which does or does not possess an upstream promoter or does or does not possess a regulatory gene, such that the threonine aldolase gene is contained in a gene structure. Plasmids or vectors which contain the threonine aldolase gene and are suitable for transforming a riboflavin producer can be obtained by cloning the threonine aldolase gene. The cells which can be obtained by transformation contain the gene in replicatable form, i.e. in additional copies in the chromosome, with the gene copies being integrated at

arbitrary sites in the genome by means of homologous recombination.

The objective, according to the invention, of partial or complete intracellular formation of glycine can also be achieved by preparing organisms in which the intracellular degradation of glycine is at least partially blocked. Mutations of this nature can, as already described above, either be generated in a random manner by means of classical methods using physical or chemical mutagenesis, for example using UV irradiation or mutation-provoking chemicals, or in a targeted manner by means of genetic engineering methods.

According to the invention, the objective of the increased intracellular formation of glycine can preferably be achieved by altering the gene for serine hydroxymethyltransferase. Such alterations can, for example, be achieved by mutations, such as insertions, deletions or substitutions, in the structural gene or the regulatory elements, such as promoters and transcription factors, which are associated with this gene.

According to the invention, it was observed, surprisingly, that these mutants include mutants which are resistant to glycine antimetabolites. The glycine antimetabolite-resistant mutants which are preferred are those unicellular or multicellular organisms which are resistant to alpha-aminomethylphosphonic acid and/or alpha-aminosulfonic acid.

This can, for example, be achieved in exactly the same way by selecting mutants which are replaced by the threonine structural analog β-hydroxynorvaline and/or which are substituted at the threonine and/or lysine analogs.

Consequently, mutants which can be employed in accordance with the invention can also be prepared by appropriate selection. Such resistant unicellular or multicellular organisms can therefore be prepared using the classical screening methods which are in general use in microbiology.

In the organisms described, riboflavin production can be further increased if the export into the medium of the glycine which is formed intracellularly is at least partially blocked. In the simplest case, it is sufficient to supplement with glycine in order to achieve this. As an alternative, the carrier which is responsible for the export can be switched off by disrupting the gene.

In addition, an increase in intracellular glycine concentration can be achieved by altering the glyoxylate metabolism, e.g. by increasing the activity of glyoxylate aminotransferase. Another option is to optimize the synthesis of intracellular glycine from carbon dioxide and ammonia.

In summary, it can be stated that the object according to the invention can preferably be solved by increasing intracellular synthesis of the glycine, at least partially blocking degradation of the glycine, at least partially inhibiting transport of the glycine out of the cell, altering the glyoxylate metabolism and optimizing glycine synthesis from ammonia and carbon dioxide. These solutions can be used as alternatives, or cumulatively or in any arbitrary combination.

An additional increase in riboflavin formation can be achieved by adding glycine to the nutrient medium.

The unicellular or multicellular organisms which are obtained in accordance with the invention may be any arbitrary cells which can be employed for biotechnological processes. Examples of these cells are fungi, yeasts, bacteria and plant and animal cells. In accordance with the invention, the cells are preferably transformed fungal cells, particularly preferably fungal cells of the genus Ashbya. The species Ashbya gossypii is particularly preferred in this context.

In that which follows, the invention is explained in more detail with the aid of examples,

without this being associated with any restriction of the invention to the subject matter of the examples:

Example 1

- Selecting a mutant which is resistant to alpha-aminomethylphosphonic acid (AMPS).

Ashbya gossypii spores were mutagenized with UV light. The spores were then added to plates treated with 70 mM alpha-aminomethylphosphonic acid. Inhibition of riboflavin formation can be recognized by the fungus forming yellow colonies without inhibition and white colonies with inhibition. Accordingly, the yellow organisms, i.e. those which were resistant to the inhibitor, were isolated. This method was used to obtain the resistant strain AMPS-NM-01, inter alia.

Experiments carried out on plates containing 200 mM AMPS demonstrated that this strain still exhibited a yellow colony color, in contrast to the starting strain, which remained completely white. In submerged culture, the mutant exhibited the same formation of riboflavin in the absence of glycine as did the wild type in the presence of glycine (cf. Figure 1).

Investigations of the specific enzymic activities of the wild type and mutant showed that serine hydroxymethyltransferase activity was reduced by 50% (Fig. 7). Since it was possible to demonstrate by feeding ^{13}C-labeled threonine that formation of serine, which is presumably catalyzed by serine hydroxymethyltransferase, takes place from glycine (Table 1), the increase in riboflavin formation can be explained by a reduction in the quantity of glycine draining off to form serine.

The composition of the minimal medium used in Table 1 is as follows:

Solution A: (100 times)	KH_2PO_4	200	g/l	pH 6.7 with KOH
Solution B:	NH_4Cl	15	g/l	
(10 times)	Asparagine	5	g/l	
	NaCl	2	g/l	
	$MgSO_4 \times 7H_2O$	4	g/l	
	$MnSO_4 \times H_2O$	0.5	g/l	
	$ClCl_2 \times 2H_2O$	0.4	g/l	
	Myoinositol	1.0	g/l	
	Nicotinamide	2.5	g/l	
	Yeast extract	2	g/l	
C source:	Glucose or soybean oil	2.5	g/l	

In order to prepare the medium, the C source was added to one-times concentrated solution B and the mixture was sterilized by autoclaving. After the medium had cooled down, 1/100 of the volume of separately autoclaved solution A was added.

Example 2
Isolation of the GLY1 gene from Ashbya gossypii

In order to isolate the gene for threonine aldolase, the glycine-auxotrophic Saccharomyces cerevisiae mutant YM 13F (SHM1 : : HIS3 shm2 : : LEU2 gly1 : : URA3) was transformed, after selection for resistance to fluoroorotic acid, with an Ashbya gossypii gene library. The gene library consisted of genomic DNA which had been partially digested with Sau3A and from which fragments of 8 - 16 kb in size had been isolated by density gradient centrifugation and ligated into the BamHI-cut vector Yep352. The transformants were first of all selected for uracil prototrophy. Selection for glycine prototrophy was carried out in a second step after replica plating. 25 glycine-prototrophic clones were isolated from about 70,000

uracil-prototrophic clones. Curing of the transformants and retransformation with the isolated plasmids demonstrated that the complementation was plasmid-encoded. Whereas there was no measurable threonine aldolase activity (< 0.1 mU/mg of protein) in the glycine-auxotrophic Saccharomyces strain, it was possible to measure significant enzyme activity (25 mU/mg of protein) in the strains which were transformed with the isolated gene library plasmids. A sub-cloned 3.7 kb Hind III fragment which exhibited complementation was sequenced (Figure 2). A threonine aldolase-encoding gene which was homologous to Saccharomyces cerevisiae GLY1 was found.

Example 3
Overexpressing the GLY1 gene in Ashbya gossypii

In order to overexpress the GLY1 gene, it was cloned into the expression vector pAG203 (cf. WO9200379). In this plasmid, the gene is under the control of the TEF promoter and the TEF terminator (Figure 3). A gene for resistance to G418 functions as a selective marker in Ashbya gossypii. After Ashbya gossypii had been transformed with this plasmid and single-spore clones had subsequently been isolated, because the spores are mononuclear and haploid, the threonine aldolase activity in the crude extract was then measured. Both when growing on glucose and when growing on soybean oil, at least ten-fold overexpression was measured in A.g.p.AG203GLY1 as compared with a strain which had been transformed with the empty plasmid pAG203 (Figure 4).

Example 4
Increasing riboflavin formation by overexpressing GLY1 and feeding threonine

Threonine was added to the medium in order to check whether the threonine which is formed in the cell limits the formation of glycine by the overexpressed threonine aldolase. When 6 grams of threonine were added per liter when A.g.pAG203GLY1 was growing on glucose as the carbon source, the strain formed approximately twice as much riboflavin as it did when 6 grams of glycine were added per liter (Figure 5). This effect was not observed when a wild type and a control strain which was transformed with the empty plasmid were tested. Analysis of the amino acids in the medium showed that only about 6 mM of the fed-in 52 mM of threonine remained in the case of the GLY1 overexpresser and that, surprisingly, the concentration of glycine had increased from 2 mM to 42 mM. These results demonstrated that glycine formation was limited by threonine, that the overexpressed threonine aldolase was capable of functioning, that glycine which was formed intracellularly was more effective than glycine which was fed extracellularly, and that the fungal cells exported glycine massively.

Example 5
Inhibiting glycine export

If the threonine aldolase-overexpressing strain A.g.pAG203GLY1 was cultured on soybean oil instead of glucose, as in Example 4, the increase in riboflavin formation obtained when feeding threonine did not exceed that when feeding glycine (Fig. 6). However, analysis of the medium showed that the threonine had been degraded down to about 13 mM. There cannot, therefore, have been any limitation in the threonine. At the same time, it was found that the extracellular glycine

had increased from 2 to about 44 mM. All the glycine which had been formed by the fungus had therefore been exported into the medium. It was possible to inhibit this export by introducing glycine into the medium, a measure which then resulted in the riboflavin formation being substantially increased in association with the same uptake of threonine (Table 2). In order to rule out the possibility that it was only the glycine which had been introduced which was responsible for the increased production, as much glycine was introduced, in a control, as was ultimately formed, as glycine, in the experiment using glycine and threonine. This finding underlines the fact that glycine which is formed intracellularly is much more effective than glycine which is added extracellularly.

Example 6

Increasing the formation of riboflavin by selecting β-hydroxynorvaline-resistant mutants

Since it was not the conversion of threonine into glycine but the synthesis of threonine which first of all limited glycine formation, the threonine analog β-hydroxynorvaline was used to search for resistant mutants. Radial growth was significantly inhibited on agar plates filled with minimum medium containing 2.5 mM β-hydroxynorvaline. Mutants which grew more vigorously formed spontaneously at the edges of the colonies. Stable mutants which grew significantly more vigorously on the β-hydroxynorvaline minimal medium than did the parental strains (Fig. 8) were produced by isolating spores and selecting once again. Investigation of riboflavin formation indicated a marked increase in productivity. First, in minimal medium containing soybean oil, the strain HNV-TB-25 formed 41 ± 11 mg of riboflavin/l whereas its parental strain only produced 18 ± 3 mg/l. The progeny strain HNV-TB-29 also exhibits a marked increase, with a formation of 116 ± 4 mg/l, as

compared with its strain of origin, i.e. Ita-GS-01, which only formed 62 ± 10 mg/l.

Table 1: ^{13}C-enrichment in the C atoms of serine, threonine and glycine following growth of A. gossypii ATC10895 on the given media and subsequent total hydrolysis of the resulting biomass (MM: minimal medium; YE: yeast extract; YNB: yeast nitrogen base; n.d.: not determined)

Medium		MM + 0.2 g of YE/l + 1 g of ethanol/l + 2.7 mg of $^{13}C_2$-serine/l	MM + 0.2 g of YNB/l + 1 g of ethanol/l + 2.6-mg of $^{13}C_1$-serine/l
Serine	C_1	1.1	4.9
	C_2	5.9	1.1
	C_3	1.1	1.1
Threonine	C_1	n.d.	39.0
	C_2		1.1
	C_3		1.1
	C_4		1.1
Glycine	C_1	1.1	7.1
	C_2	4.3	1.1

Table 2: Effect of supplementation with threonine and glycine on riboflavin formation when GLY1 is simultaneously being overexpressed

Strain	Carbon source	t = 0 Supplement	t = 72 h Riboflavin [mg/l]	t = 72 h Gly [mM]	t = 72 h Thr [mM]
WT	Soybean oil	80 mM Gly 50 mM Thr	22 ± 1	80 ± 2	42 ± 0
		130 mM Gly	18 ± 3	129 ± 2	n.d.
	Glucose	80 mM Gly 50 mM Thr	5 ± 1	80 ± 0	35 ± 0
		130 mM Gly	7 ± 1	126 ± 2	n.d.
Ag pAG 203 GLY1	Soybean oil	80 mM Gly 50 mM Thr	31 ± 0	117 ± 2	11 ± 1
		130 mM Gly	20 ± 3	129 ± 1	n.d.
	Glucose	80 mM Gly 50 mM Thr	40 ± 1	113 ± 2	12 ± 0.7
		130 mM Gly	9 ± 1	129 ± 3	n.d.

n.d. = not detectable

Comments on the figures

Figure 1: Formation of riboflavin by the Ashbya gossypii strains ATCC 10895 (wild type, WT) and the AMPS-resistant mutant AMPS-MN-01 in the presence or absence of 6 g of glycine/l following growth on complete medium containing 10 g of soybean oil/l as the carbon source. The measured values were obtained from three independent experiments.

Figure 2a: Gly 1 locus in the Ashbya gossypii genome. The clones GB 7-1 and GB 26-9, and also the 3.7 kb Hind III subclone GB-26-9-6, complement the S. cerevisiae mutant. GB-26-9-6 was sequenced entirely while GB 7-1 was sequenced in order to complete the C terminus of GLY1.

Figure 2b: Nucleotide sequence, and deduced amino acid sequence, of the A. gossypii GLY1 gene together with the flanking nucleotide sequence.

Figure 3: Diagrammatic depiction of the construction of the vector pAG203GLY1 for overexpressing the GLY1 gene in A. gossypii.

Figure 4: Comparison of Ashbya gossypii wild type (solid symbols) and A.g.pAG203GLY1 (open symbols) with regard to growth, riboflavin formation and specific threonine aldolase activity when cultured on complete medium containing 10 g of soybean oil/l.

Figure 5: Growth and riboflavin formation of Ashbya gossypii strains ATCC 10895 (wild type), pAG203 and pAG203GLY1 when cultured on YE

complete medium containing 10 g of glucose/l as the carbon source and in association with glycine or threonine supplementation. The Table shows the glycine and threonine concentrations in the medium in each case before and after culture. The mean values and standard deviations shown represent the results from three independent experiments.

Figure 6: Growth and riboflavin formation of Ashbya gossypii strains ATCC 10895 (wild type), pAG203 and pAG203GLY1 when cultured on complete medium containing 10 g of glucose/l as the C source and in association with glycine or threonine supplementation. The Table shows the glycine and threonine concentrations in the medium in each case before and after culture. The mean values and standard deviations shown represent the results from three independent experiments.

Figure 7: Comparison of Ashbya gossypii wild type (solid symbols) and the AMPS-resistant mutant AMPS-NM-01 with regard to growth, riboflavin formation and the specific activities of threonine aldolase, serine hydroxymethyltransferase and glutamate glyoxylate aminotransferase when cultured on complete medium containing 10 g of soybean oil/l. The measured values were obtained from three independent experiments.

Figure 8: Effect of β-hydroxynorvaline on Ashbya gossypii; growth of wild type (W) and HNV-TB-25 (H) on an agar plate which is filled with minimal medium containing 2.5 g of glucose/l and 2.5 mM β-hydroxynorvaline.

SEQUENCE LISTING

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(ii) TITLE OF INVENTION: UNICELLULAR OR MULTICELLULAR ORGANISMS FOR PREPARING RIBOFLAVIN

(iii) NUMBER OF SEQUENCES: 2

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(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Disk 3.5" / 1.44 MB
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: TXT ASCII

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: 2,255,284
(B) FILING DATE: December 17, 1998

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: DE 197 57 180.8
(B) FILING DATE: December 22, 1997

(A) APPLICATION NUMBER: DE 198 40 709.2
(B) FILING DATE: September 09, 1998

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2744 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Ashbya gossypii

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1232..2377
(C) IDENTIFICATION METHOD: experimental
(D) OTHER INFORMATION:/codon_start= 1232
/product= "Threonin-Aldolase"
/evidence= EXPERIMENTAL
/number= 1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TTGCCATTAA TGACCGGGAG CCTGAAGGTG TGTGATGAAC AAGCCAGTCT TCCCCGCGCG 60

TCGCCAACTG CTCGTCATAT AATCCCGGAA AAGCTCGCAT TAGGTGAAAT TTTTCTTAGG 120

AATTACATCT GCTACTGACA AAACTAAGTA AAAGCTCCGA TAGGTAGCCG TGCTGCCGAG 180

CACCTGCCTA ATACACGCAG GCGCCATACA CTATTTAAGC ACAATGTTAT CGCCCCGCAG 240

CTTGAGGTAT TCCTGGTCGA TGCCAGGTGT CATAGGCTTG ATCACCAGCG AGTAGACCTC 300

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ACTATTGTAG AAGCGCAGCC CGTTGCTGGG GGACTTGTAG CGCGCCTTGA GCCCCGTGAT      360

GTCGCAGTAG CGTTTCACGG GATACTGCGA TGGTGGCGCC TGAATGTTGA AGTATGTCAG      420

CTTCGTGCGC CCTGCGTCAC GCCCGGCTTC CGACTGTGCC TCTGTCGTGA GCCGTTTCCA      480

CTCGTCTGTC AGAAGCTGAC GTGTCGGCTT GTGGCGGCGC GTGGGTTTCT TCCACGTGGG      540

CGACTTGAAG TCGCTACGAC TGGTATCATT ACGTGCTGCA ATCGCTCGGA GGTTCTCCAT      600

CTGGGGTCCA CGGTCGCTCG TTGATCTGTC TATCTCGAAA TCCCTGCCCA GATGTACTCC      660

CATGTTATCA CGTGACCACA CGCCGTTTTC GTGTGTAGTG ATGCAGATGG TTCTAGAGCA      720

TCACGTGGCT TACATAGCTT TGTTACATAA TCGATTTTCC GCAGGAGCGT TACGTCCAAC      780

GGTCGTTCTG TGCCAAAAGC AACAACTGAG CGTCAGGCGG CCGTCTCCCC AGACACGCTC      840

CGCCCCAAAC TGAGCTCCAC GCGGCCTTCT GTCCGAGTTA AGTTCCTCCC CGCTCGTCAG      900

CACGGGGTCT TTCGTCGCCT ATCCTCCTGC AGCGTTCGCT ACTGCAGATC GTGAGCAGTG      960

GCACCCGCGA CCAAAAAAAG AAATTATGTT CCTTACGCAA GGAATATGCC TCGCGCCATG     1020

CCATCGCAAA GAGTGATGCC GCAGAGGTTG CTTCTGCGAG GCAACTCCTG GGCAATAGGG     1080

TGGAAAATTC AGCTTGGGCT TATATAAAAG AAACCGTTCG AGCTCGTCGG AGCCAGGTGG     1140

AAAATTTTTC GTAACGTAGG TAGAGGTTAT AGTTAGCGTC AGTCTCTTTT CTGCCAAGCT     1200

GCTACAGTTG ACTACAAGTA ACAAACCCAG G ATG AAT CAG GAT ATG GAA CTA         1252
                                    Met Asn Gln Asp Met Glu Leu
                                      1               5

CCA GAG GCG TAC ACG TCG GCT TCG AAC GAC TTC CGT TCG GAC ACG TTC       1300
Pro Glu Ala Tyr Thr Ser Ala Ser Asn Asp Phe Arg Ser Asp Thr Phe
         10                  15                  20

ACC ACT CCA ACG CGC GAA ATG ATC GAG GCT GCG CTA ACG GCG ACC ATC       1348
Thr Thr Pro Thr Arg Glu Met Ile Glu Ala Ala Leu Thr Ala Thr Ile
     25                  30                  35

GGT GAC GCC GTC TAC CAA GAG GAC ATC GAC ACG TTG AAG CTA GAA CAG       1396
Gly Asp Ala Val Tyr Gln Glu Asp Ile Asp Thr Leu Lys Leu Glu Gln
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40                  45                  50                  55

CAC GTC GCC AAG CTG GCC GGC ATG GAG GCC GGT ATG TTC TGC GTA TCT      1444
His Val Ala Lys Leu Ala Gly Met Glu Ala Gly Met Phe Cys Val Ser
                60                  65                  70

GGT ACT TTG TCC AAC CAG ATT GCT TTG CGG ACC CAC CTA ACT CAG CCA      1492
Gly Thr Leu Ser Asn Gln Ile Ala Leu Arg Thr His Leu Thr Gln Pro
            75                  80                  85

CCA TAT TCG ATT CTT TGC GAC TAC CGT GCG CAT GTG TAC ACG CAC GAG      1540
Pro Tyr Ser Ile Leu Cys Asp Tyr Arg Ala His Val Tyr Thr His Glu
        90                  95                  100

GCT GCG GGG TTG GCA ATT TTG TCC CAG GCC ATG GTG ACA CCT GTC ATT      1588
Ala Ala Gly Leu Ala Ile Leu Ser Gln Ala Met Val Thr Pro Val Ile
    105                 110                 115

CCT TCC AAC GGC AAC TAC TTG ACT TTG GAA GAC ATC AAG AAG CAC TAC      1636
Pro Ser Asn Gly Asn Tyr Leu Thr Leu Glu Asp Ile Lys Lys His Tyr
120                 125                 130                 135

ATT CCT GAT GAT GGC GAC ATC CAC GGT GCT CCA ACA AAG GTT ATC TCG      1684
Ile Pro Asp Asp Gly Asp Ile His Gly Ala Pro Thr Lys Val Ile Ser
                140                 145                 150

TTG GAA AAC ACC TTG CAC GGT ATC ATT CAC CCA CTA GAG GAG CTT GTT      1732
Leu Glu Asn Thr Leu His Gly Ile Ile His Pro Leu Glu Glu Leu Val
            155                 160                 165

CGG ATC AAG GCT TGG TGT ATG GAG AAC GAC CTC AGA CTA CAC TGC GAT      1780
Arg Ile Lys Ala Trp Cys Met Glu Asn Asp Leu Arg Leu His Cys Asp
        170                 175                 180

GGT GCG AGA ATC TGG AAC GCG TCC GCA GAA TCC GGT GTG CCT CTA AAA      1828
Gly Ala Arg Ile Trp Asn Ala Ser Ala Glu Ser Gly Val Pro Leu Lys
    185                 190                 195

CAG TAC GGA GAG CTA TTC GAC TCC ATT TCC ATC TGC TTG TCC AAG TCC      1876
Gln Tyr Gly Glu Leu Phe Asp Ser Ile Ser Ile Cys Leu Ser Lys Ser
200                 205                 210                 215

ATG GGT GCC CCA ATG GGC TCC ATT CTC GTC GGG TCG CAC AAG TTC ATA      1924
Met Gly Ala Pro Met Gly Ser Ile Leu Val Gly Ser His Lys Phe Ile
                220                 225                 230
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            235                 240                 245

TCT GGT ATG ATG TGC AAG ATG GCG ATG GTG GCT ATC CAG GGT GAC TGG      2020
Ser Gly Met Met Cys Lys Met Ala Met Val Ala Ile Gln Gly Asp Trp
        250                 255                 260

AAG GGC AAG ATG AGG CGT TCG CAC AGA ATG GCT CAC GAG CTG GCC AGA      2068
Lys Gly Lys Met Arg Arg Ser His Arg Met Ala His Glu Leu Ala Arg
    265                 270                 275

TTT TGC GCA GAG CAC GGC ATC CCA TTG GAG TCG CCT GCT GAC ACC AAC      2116
Phe Cys Ala Glu His Gly Ile Pro Leu Glu Ser Pro Ala Asp Thr Asn
280                 285                 290                 295

TTT GTC TTT TTG GAC TTG CAG AAG AGC AAG ATG AAC CCT GAC GTG CTC      2164
Phe Val Phe Leu Asp Leu Gln Lys Ser Lys Met Asn Pro Asp Val Leu
                300                 305                 310

GTC AAG AAG AGT TTG AAG TAC GGC TGC AAG CTA ATG GGC GGG CGT GTC      2212
Val Lys Lys Ser Leu Lys Tyr Gly Cys Lys Leu Met Gly Gly Arg Val
            315                 320                 325

TCC TTC CAC TAC CAG ATA TCT GAG GAG TCC CTT GAG AAG ATC AAG CAG      2260
Ser Phe His Tyr Gln Ile Ser Glu Glu Ser Leu Glu Lys Ile Lys Gln
        330                 335                 340

GCC ATC CTA GAG GCG TTC GAG TAC TCG AAG AAG AAC CCT TAC GAT GAA      2308
Ala Ile Leu Glu Ala Phe Glu Tyr Ser Lys Lys Asn Pro Tyr Asp Glu
    345                 350                 355

AAC GGC CCC ACG AAG ATC TAC AGA AGT GAG TCC GCT GAC GCT GTG GGT      2356
Asn Gly Pro Thr Lys Ile Tyr Arg Ser Glu Ser Ala Asp Ala Val Gly
360                 365                 370                 375

GAG ATC AAG ACC TAC AAG TAT TAAGGGATTT CGATGATGAC ATGAAAAATT         2407
Glu Ile Lys Thr Tyr Lys Tyr
            380

ACATATTGGC ACGGCATAGG CATTGGGTAA TATTAAGCAT ATGGTTGAGA TGAATTACTG    2467

TTCGGGTACC GGTATTTCCA AAGTGCTGTC GACTTTTGCA AGAGATGGCT ATGAATGGGG    2527
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CACGCTCCAT CACCTCTCTG CGAGCCGGAC TCAGCATTAT ATCCATCTCA AAACCTAATA 2587

TCAAATGGGA TTGTGGTGCG CAGTACATGC GCAGTGCTGC ACATTTGAGG ATCAATGGGT 2647

TTTTCCAGGC ACTGCCTGGG TCACTCACCC TATTGCGGAG GGACTAGTAG CTCTACCATT 2707

CTGAGCTGAC TAAAATGTTT GATTCTTTTG GTACTTA 2744

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 382 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met Asn Gln Asp Met Glu Leu Pro Glu Ala Tyr Thr Ser Ala Ser Asn
1 5 10 15

Asp Phe Arg Ser Asp Thr Phe Thr Thr Pro Thr Arg Glu Met Ile Glu
20 25 30

Ala Ala Leu Thr Ala Thr Ile Gly Asp Ala Val Tyr Gln Glu Asp Ile
35 40 45

Asp Thr Leu Lys Leu Glu Gln His Val Ala Lys Leu Ala Gly Met Glu
50 55 60

Ala Gly Met Phe Cys Val Ser Gly Thr Leu Ser Asn Gln Ile Ala Leu
65 70 75 80

Arg Thr His Leu Thr Gln Pro Pro Tyr Ser Ile Leu Cys Asp Tyr Arg
85 90 95

Ala His Val Tyr Thr His Glu Ala Ala Gly Leu Ala Ile Leu Ser Gln
100 105 110

Ala Met Val Thr Pro Val Ile Pro Ser Asn Gly Asn Tyr Leu Thr Leu
115 120 125

Glu Asp Ile Lys Lys His Tyr Ile Pro Asp Asp Gly Asp Ile His Gly

```
    130                 135                 140

Ala Pro Thr Lys Val Ile Ser Leu Glu Asn Thr Leu His Gly Ile Ile
145                 150                 155                 160

His Pro Leu Glu Glu Leu Val Arg Ile Lys Ala Trp Cys Met Glu Asn
                165                 170                 175

Asp Leu Arg Leu His Cys Asp Gly Ala Arg Ile Trp Asn Ala Ser Ala
            180                 185                 190

Glu Ser Gly Val Pro Leu Lys Gln Tyr Gly Glu Leu Phe Asp Ser Ile
        195                 200                 205

Ser Ile Cys Leu Ser Lys Ser Met Gly Ala Pro Met Gly Ser Ile Leu
    210                 215                 220

Val Gly Ser His Lys Phe Ile Lys Lys Ala Asn His Phe Arg Lys Gln
225                 230                 235                 240

Gln Gly Gly Gly Val Arg Gln Ser Gly Met Met Cys Lys Met Ala Met
                245                 250                 255

Val Ala Ile Gln Gly Asp Trp Lys Gly Lys Met Arg Arg Ser His Arg
            260                 265                 270

Met Ala His Glu Leu Ala Arg Phe Cys Ala Glu His Gly Ile Pro Leu
        275                 280                 285

Glu Ser Pro Ala Asp Thr Asn Phe Val Phe Leu Asp Leu Gln Lys Ser
    290                 295                 300

Lys Met Asn Pro Asp Val Leu Val Lys Lys Ser Leu Lys Tyr Gly Cys
305                 310                 315                 320

Lys Leu Met Gly Gly Arg Val Ser Phe His Tyr Gln Ile Ser Glu Glu
                325                 330                 335

Ser Leu Glu Lys Ile Lys Gln Ala Ile Leu Glu Ala Phe Glu Tyr Ser
            340                 345                 350

Lys Lys Asn Pro Tyr Asp Glu Asn Gly Pro Thr Lys Ile Tyr Arg Ser
        355                 360                 365

Glu Ser Ala Asp Ala Val Gly Glu Ile Lys Thr Tyr Lys Tyr
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370 375 380

Claims

1. A unicellular or multicellular organism, in particular a microorganism, for the biotechnological preparation of riboflavin, which exhibits a glycine metabolism which is altered such that its synthetic output of riboflavin without any external supply of glycine is at least equal to that of a wild type of the species Ashbya gossypii, i.e. ATCC10895, which is cultured under standard conditions with the addition of 6 g of external glycine/l.

2. A unicellular or multicellular organism as claimed in claim 1, in which the intracellular synthesis of glycine is increased and/or the intracellular degradation of glycine and/or the transport of glycine out of the cell is at least partially inhibited.

3. A unicellular or multicellular organism as claimed in claim 1 or 2, which exhibits an increased threonine aldolase activity.

4. A unicellular or multicellular organism as claimed in one of claims 1 to 3, in which the intracellular formation of serine from glycine is at least partially blocked.

5. A unicellular or multicellular organism as claimed in claim 4, in which the activity of serine hydroxymethyltransferase is at least partially blocked.

6. A unicellular or multicellular organism as claimed in claim 4 or 5, which is resistant to glycine antimetabolites.

7. A unicellular or multicellular organism as claimed in claim 6, which is resistant to alpha-aminomethylphosphonic acid or alpha-aminosulfonic acid, β-hydroxynorvaline and/or other threonine and/or lysine analogs.

8. A unicellular or multicellular organism as claimed in any one of claims 1 to 7, which is a fungus, preferably from the genus Ashbya.

9. A unicellular or multicellular organism as claimed in one of claims 1 to 8, which is a fungus of the species Ashbya gossypii.

10. A threonine aldolase gene having a nucleotide sequence which encodes the amino acid sequence shown in Figure 2b and its allelic variantion.

11. A threonine aldolase gene as claimed in claim 10 which has the nucleotide sequence of nucleotide 1 to 1149 as depicted in Fig. 2b or a DNA sequence which has essentially the same effect.

12. A threonine aldolase gene as claimed in claim 10 or 11 which possesses an upstream promoter having the nucleotide sequence from nucleotide -1231 to -1 as depicted in Fig. 2b or a DNA sequence which has essentially the same effect.

13. A threonine aldolase gene as claimed in one of claims 10 to 12 together with regulatory gene sequences which are assigned to this gene.

14. A gene structure which contains a threonine aldolase gene as claimed in one of claims 10 to 13.

15. A vector which contains a threonine aldolase gene as claimed in one of claims 10 to 13 or a gene structure as claimed in claim 14.

16. A transformed organism for preparing riboflavin which contains, in replicatable form, a threonine aldolase gene as claimed in one of claims 10 to 13 or a gene structure as claimed in claim 14.

17. A transformed organism as claimed in claim 16 which contains a vector as claimed in claim 15.

18. A process for preparing riboflavin, which comprises employing an organism as claimed in one of claims 1 to 9.

19. A process for preparing a riboflavin-producing unicellular or multicellular organism which comprises altering the organism such that it exhibits a glycine metabolism which is altered such that its synthetic output of riboflavin without any external supply of glycine is at least equal to that of a wild type of the

species Ashbya gossypii, i.e. ATCC10895, which is cultured under standard conditions with the addition of 6 g of external glycine/l.

20. The process as claimed in claim 19, wherein the organism is altered using genetic engineering methods.

21. The process as claimed in claim 19 or 20, wherein the alteration of the organism is achieved by exchanging the promoter and/or increasing the gene copy number.

22. The process as claimed in one of claims 19 to 21, wherein an enzyme possessing increased activity is produced by altering the endogenous threonine aldolase gene.

23. The process as claimed in one of claims 19 to 22, wherein the activity of the serine hydroxymethyltransferase is at least partially blocked by altering the endogenous serine hydroxymethyltransferase gene.

24. The use of the organism as claimed in one of claims 1 to 9 and 16 and 17 for preparing riboflavin.

25. The use of the threonine aldolase gene as claimed in one of claims 10 to 13 and the gene structure as claimed in claim 14 for preparing an organism as claimed in one of claims 1 to 9 and 16 and 17.

26. The use of the vector as claimed in claim 15 for preparing an organism as claimed in one of claims 1 to 9 and 16 and 17.

Drawings

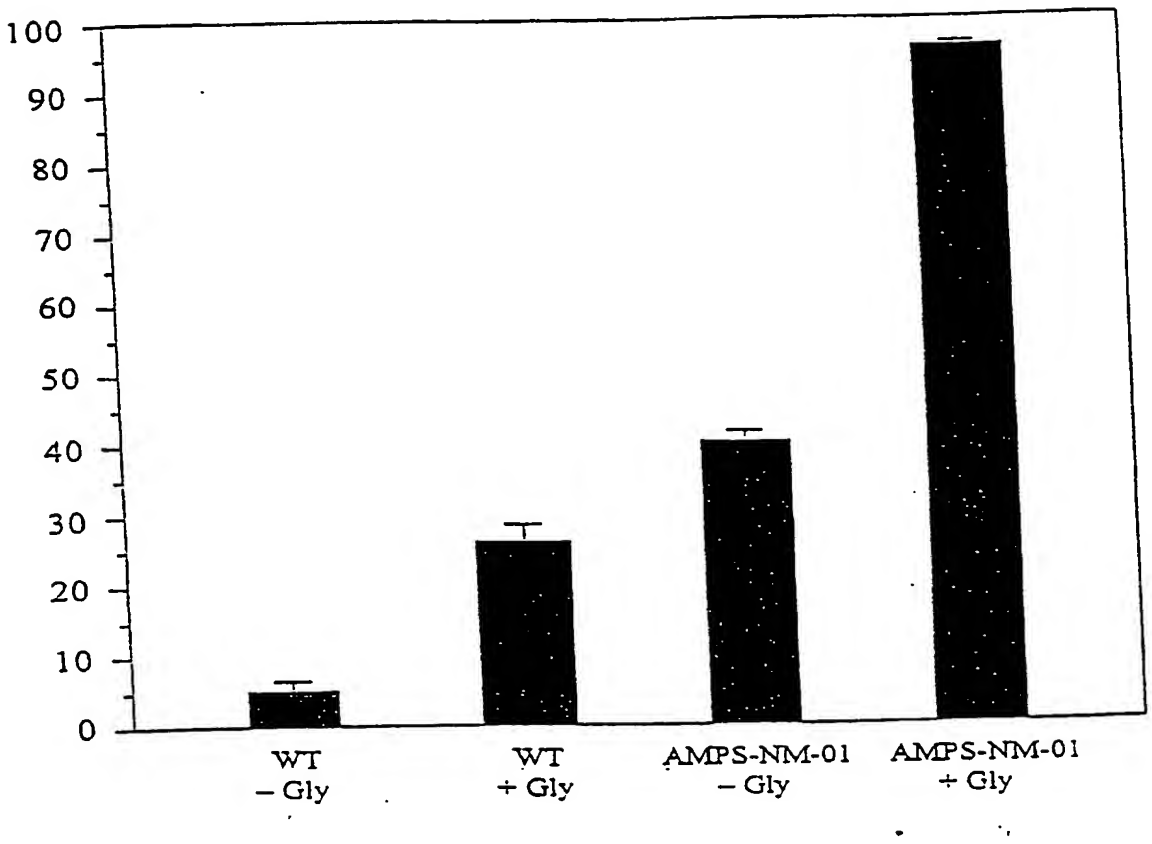

Fig. 1

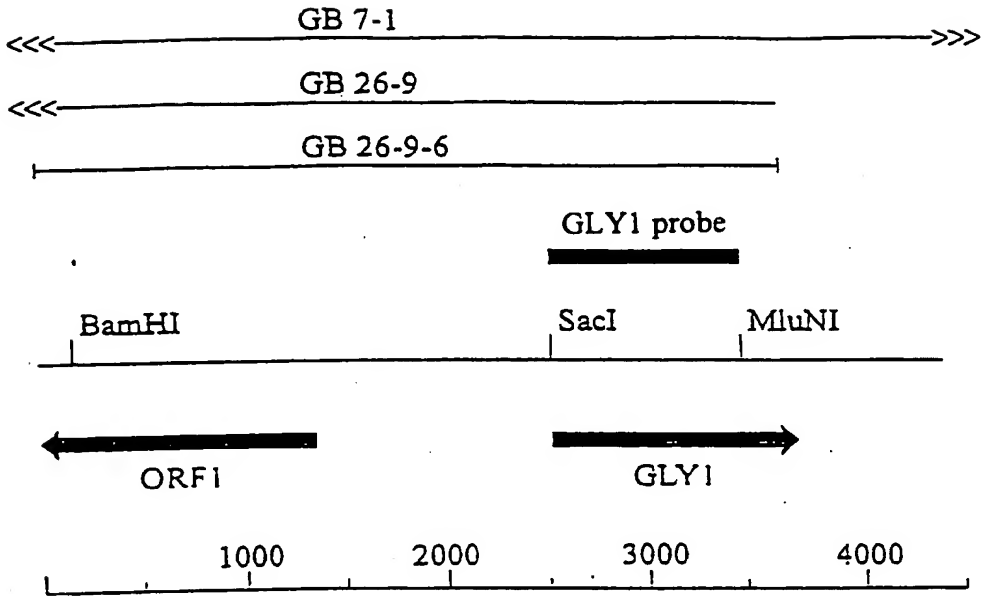
GB 7-1
GB 26-9
GB 26-9-6
GLY1 probe
BamHI
SacI
MluNI
ORF1
GLY1
1000
2000
3000
4000

Fig. 2a

Sequences

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GTGATGAACA AGCCAGTCTT CCCCGCGCGT CGCCAACTGC TCGTCATATA ATCCCGGAAA -1141
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AAGCTCCGAT AGGTAGCCGT GCTGCCGAGC ACCTGCCTAA TACACGCAGG CGCCATACAC -1021
TATTTAAGCA CAATGTTATC GCCCCGCAGC TTGAGGTATT CCTGGTCGAT GCCAGGTGTC  -961
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GACTTGTAGC GCGCCTTGAG CCCCGTGATG TCGCAGTAGC GTTTCACGGG ATACTGCGAT  -841
GGTGGCGCCT GAATGTTGAA GTATGTCAGC TTCGTGCGCC CTGCGTCACG CCCGGCTTCC  -781
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TGGCGGCGCG TGGGTTTCTT CCACGTGGGC GACTTGAAGT CGCTACGACT GGTATCATTA  -661
CGTGCTGCAA TCGCTCGGAG GTTCTCCATC TGGGGTCCAC GGTCGCTCGT TGATCTGTCT  -601
ATCTCGAAAT CCCTGCCCAG ATGTACTCCC ATGTTATCAC GTGACCACAC GCCGTTTTCG  -541
TGTGTAGTGA TGCAGATGGT TCTAGAGCAT CACGTGGCTT ACATAGCTTT GTTACATAAT  -481
CGATTTTCCG CAGGAGCGTT ACGTCCAACG GTCGTTCTGT GCCAAAAGCA ACAACTGAGC  -421
GTCAGGCGGC CGTCTCCCCA GACACGCTCC GCCCCAAACT GAGCTCCACG CGGCCTTCTG  -361
TCCGAGTTAA GTTCCTCCCC GCTCGTCAGC ACGGGGTCTT TCGTCGCCTA TCCTCCTGCA  -301
GCGTTCGCTA CTGCAGATCG TGAGCAGTGG CACCCGCGAC CAAAAAAAGA AATTATGTTC  -241
CTTACGCAAG GAATATGCCT CGCGCCATGC CATCGCAAAG AGTGATGCCG CAGAGGTTGC  -181
TTCTGCGAGG CAACTCCTGG GCAATAGGGT GGAAAATTCA GCTTGGGCTT ATATAAAAGA  -121
AACCGTTCGA GCTCGTCGGA GCCAGGTGGA AAATTTTTCG TAACGTAGGT AGAGGTTATA   -61
GTTAGCGTCA GTCTCTTTTC TGCCAAGCTG CTACAGTTGA CTACAAGTAA CAAACCCAGG    -1

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 1 Met Asn Gln Asp Met Glu Leu Pro Glu Ala Tyr Thr Ser Ala Ser Asn

   GAC TTC CGT TCG GAC ACG TTC ACC ACT CCA ACG CGC GAA ATG ATC GAG    96
17 Asp Phe Arg Ser Asp Thr Phe Thr Thr Pro Thr Arg Glu Met Ile Glu

   GCT GCG CTA ACG GCG ACC ATC GGT GAC GCC GTC TAC CAA GAG GAC ATC   144
33 Ala Ala Leu Thr Ala Thr Ile Gly Asp Ala Val Tyr Gln Glu Asp Ile

   GAC ACG TTG AAG CTA GAA CAG CAC GTC GCC AAG CTG GCC GGC ATG GAG   192
49 Asp Thr Leu Lys Leu Glu Gln His Val Ala Lys Leu Ala Gly Met Glu

   GCC GGT ATG TTC TGC GTA TCT GGT ACT TTG TCC AAC CAG ATT GCT TTG   240
65 Ala Gly Met Phe Cys Val Ser Gly Thr Leu Ser Asn Gln Ile Ala Leu

   CGG ACC CAC CTA ACT CAG CCA CCA TAT TCG ATT CTT TGC GAC TAC CGT   288
81 Arg Thr Gly Leu Thr Gln Pro Pro Tyr Ser Ile Leu Cys Asp Tyr Arg

   GCG CAT GTG TAC ACG CAC GAG GCT GCG GGG TTG GCA ATT TTG TCC CAG   336
97 Ala His Val Tyr Thr His Glu Ala Ala Gly Leu Ala Ile Leu Ser Gln

   GCC ATG GTG ACA CCT GTC ATT CCT TCC AAC GGC AAC TAC TTG ACT TTG   384
113 Ala Met Val Thr Pro Val Ile Pro Ser Asn Gly Asn Tyr Leu Thr Leu

   GAA GAC ATC AAG AAG CAC TAC ATT CCT GAT GAT GGC GAC ATC CAC GGT   432
129 Glu Asp Ile Lys Lys His Tyr Ile Pro Asp Asp Gly Asp Ile His Gly

   GCT CCA ACA AAG GTT ATC TCG TTG GAA AAC ACC TTG CAC GGT ATC ATT   480
145 Ala Pro Thr Lys Val Ile Ser Leu Glu Asn Thr Leu His Gly Ile Ile

   CAC CCA CTA GAG GAG CTT GTT CGG ATC AAG GCT TGG TGT ATG GAG AAC   528
161 His Pro Leu Glu Glu Leu Val Arg Ile Lys Ala Trp Cys Met Glu Asn

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177 Asp Leu Arg Leu His Cys Asp Gly Ala Arg Ile Trp Asn Ala Ser Ala

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193 Glu Ser Gly Val Pro Leu Lys Gln Tyr Gly Glu Leu Phe Asp Ser Ile

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241 Gln Gly Gly Gly Val Arg Gln Ser Gly Met Met Cys Lys Met Ala Met

    GTG GCT ATC CAG GGT GAC TGG AAG GGC AAG ATG AGG CGT TCG CAC AGA   816
257 Val Ala Ile Gln Gly Asp Trp Lys Gly Lys Met Arg Arg Ser His Arg

    ATG GCT CAC GAG CTG GCC AGA TTT TGC GCA GAG CAC GGC ATC CCA TTG   864
273 Met Ala His Glu Leu Ala Arg Phe Cys Ala Glu His Gly Ile Pro Leu

    GAG TCG CCT GCT GAC ACC AAC TTT GTC TTT TTG GAC TTG CAG AAG AGC   912
289 Glu Ser Pro Ala Asp Thr Asn Phe Val Phe Leu Asp Leu Gln Lys Ser

    AAG ATG AAC CCT GAC GTG CTC GTC AAG AAG AGT TTG AAG TAC GGC TGC   960
305 Lys Met Asn Pro Asp Val Leu Val Lys Lys Ser Leu Lys Tyr Gly Cys

    AAG CTA ATG GGC GGG CGT GTC TCC TTC CAC TAC CAG ATA TCT GAG GAG  1008
321 Lys Leu Met Gly Gly Arg Val Ser Phe His Tyr Gln Ile Ser Glu Glu

    TCC CTT GAG AAG ATC AAG CAG GCC ATC CTA GAG GCG TTC GAG TAC TCG  1056
337 Ser Leu Glu Lys Ile Lys Gln Ala Ile Leu Glu Ala Phe Glu Tyr Ser

    AAG AAG AAC CCT TAC GAT GAA AAC GGC CCC ACG AAG ATC TAC AGA AGT  1104
353 Lys Lys Asn Pro Tyr Asp Glu Asn Gly Pro Thr Lys Ile Tyr Arg Ser

    GAG TCC GCT GAC GCT GTG GGT GAG ATC AAG ACC TAC AAG TAT TAA      1149
369 Glu Ser Ala Asp Ala Val Gly Glu Ile Lys Thr Tyr Lys Tyr

GGGATTTCGA TGATGACATG AAAAATTACA TATTGGCACG GCATAGGCAT TGGGTAATAT  1209
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GCATTATATC CATCTCAAAA CCTAATATCA AATGGGATTG TGGTGCGCAG TACATGCGCA  1389
GTGCTGCACA TTTGAGGATC AATGGGTTTT TCCAGGCACT GCCTGGGTCA CTCACCCTAT  1449
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CTTA
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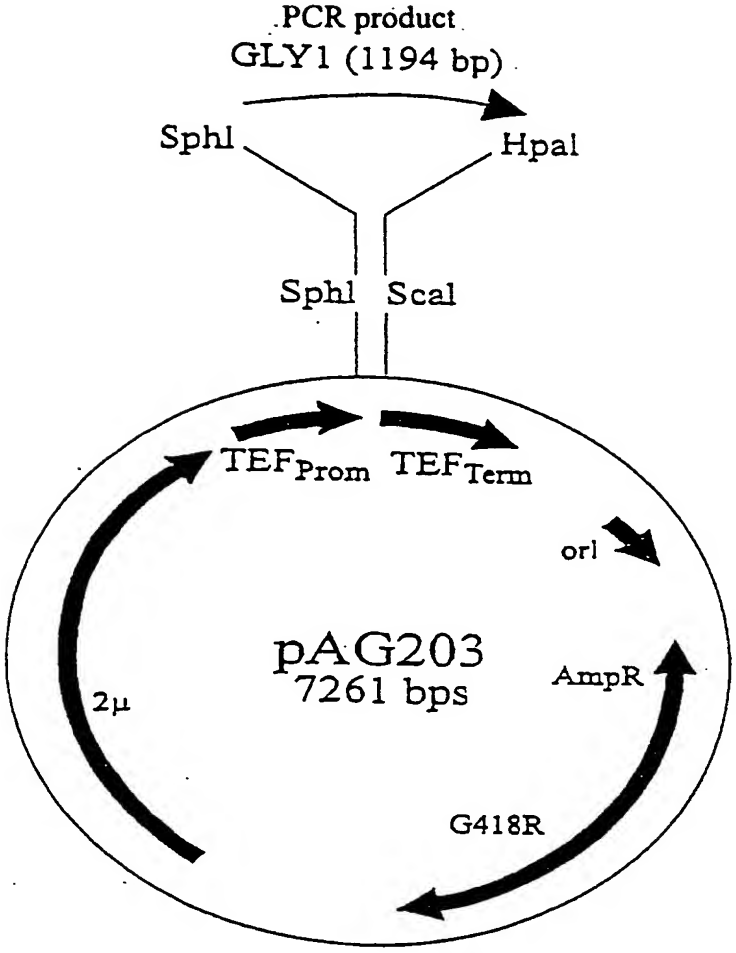
Figure 2b


PCR product
GLY1 (1194 bp)
SphI
HpaI
SphI
ScaI
TEFProm
TEFTerm
ori
pAG203
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AmpR
2μ
G418R

Fig. 3

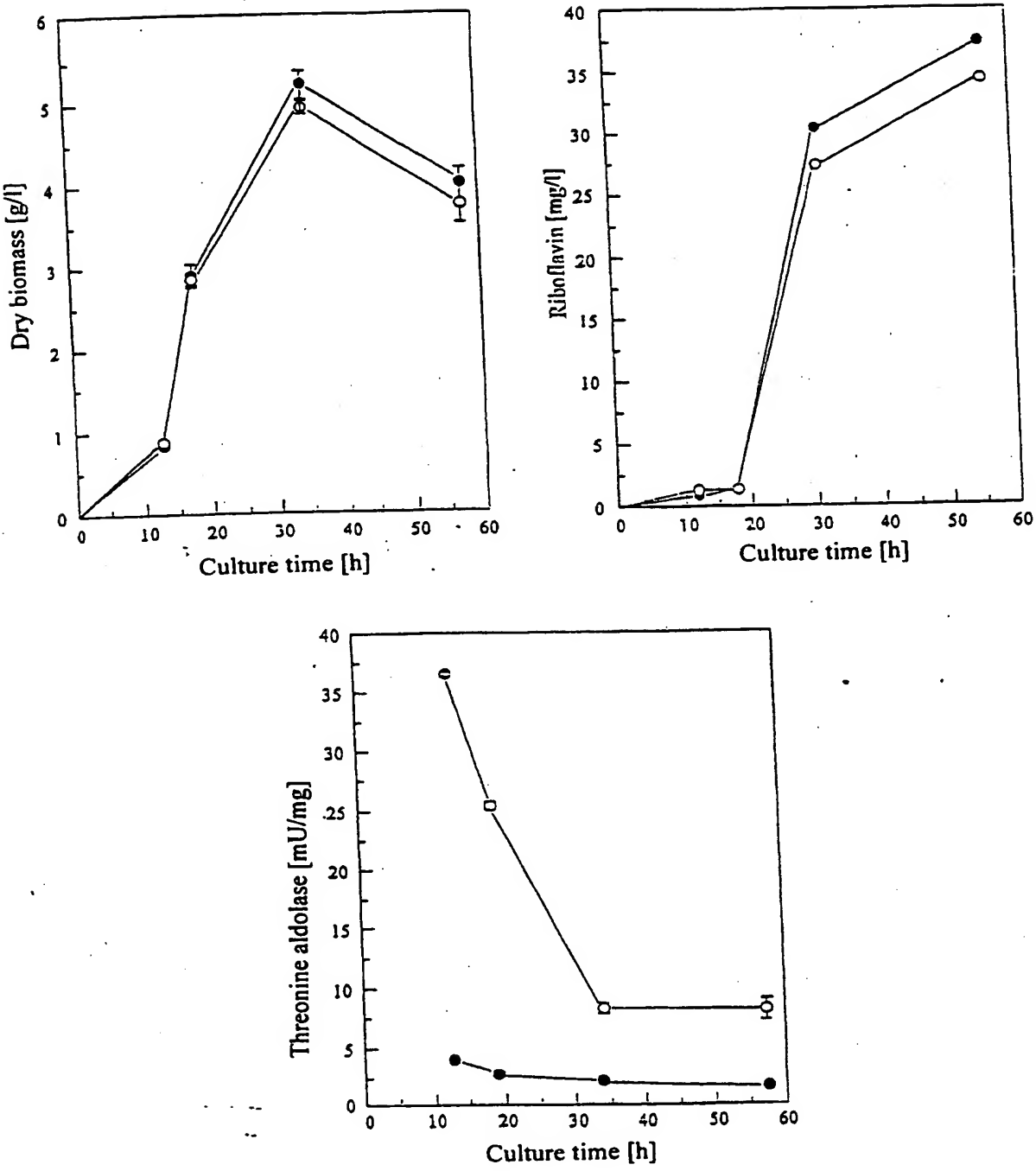
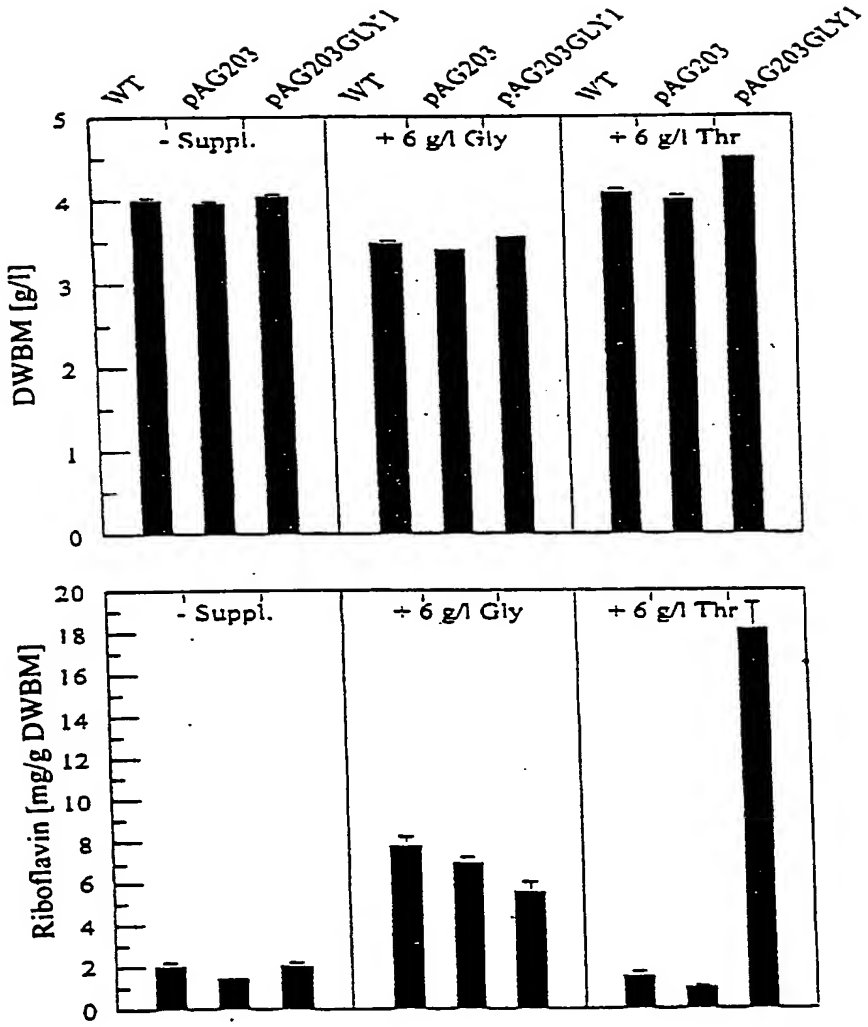
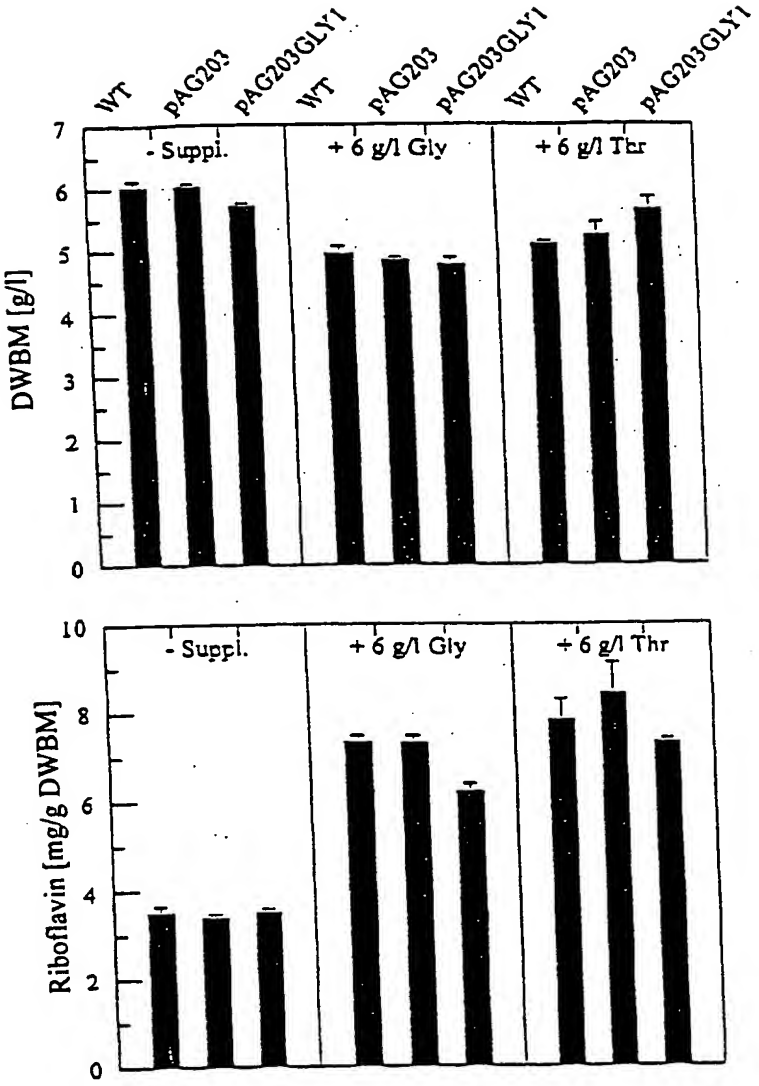

Dry biomass [g/l]
Culture time [h]
Riboflavin [mg/l]
Culture time [h]
Threonine aldolase [mU/mg]
Culture time [h]

Fig. 4



Strain	Medium	Before culture Gly [mM]	Before culture Thr [mM]	After culture Gly [mM]	After culture Thr [mM]
A. g. WT	-	2	1.6	2.3 ± 0.04	0.18 ± 0.08
	+ 6 g of Gly/l	82	1.6	79.6 ± 0.8	1.2 ± 0.1
	+ 6 g of Thr/l	2	51.6	6.3 ± 0.3	32.0 ± 1.2
A. g. pAG203GLY1	-	2	1.6	4.0 ± 0.08	0.14 ± 0.01
	+ 6 g of Gly/l	82	1.6	80.2 ± 0.7	0 ± 0
	+ 6 g of Thr/l	2	51.6	41.3 ± 0.7	6.1 ± 1.0

Fig. 5



Strain	Medium	Before culture Gly [mM]	Before culture Thr [mM]	After culture Gly [mM]	After culture Thr [mM]
A. g. ATCC 10895	-	2	1.6	2.4 ± 0.03	0 ± 0
	+ 6 g of Gly/l	82	1.6	76.5 ± 0.4	0 ± 0
	+ 6 g of Thr/l	2	51.6	5.6 ± 0.7	42.8 ± 1.0
A. g. pAG203GLY1	-	2	1.6	4.0 ± 0.06	0 ± 0
	+ 6 g of Gly/l	82	1.6	78.3 ± 2.0	0 ± 0
	+-6 g of Thr/l	2	51.6	44.0 ± 4.1	12.6 ± 1.8

Fig. 6

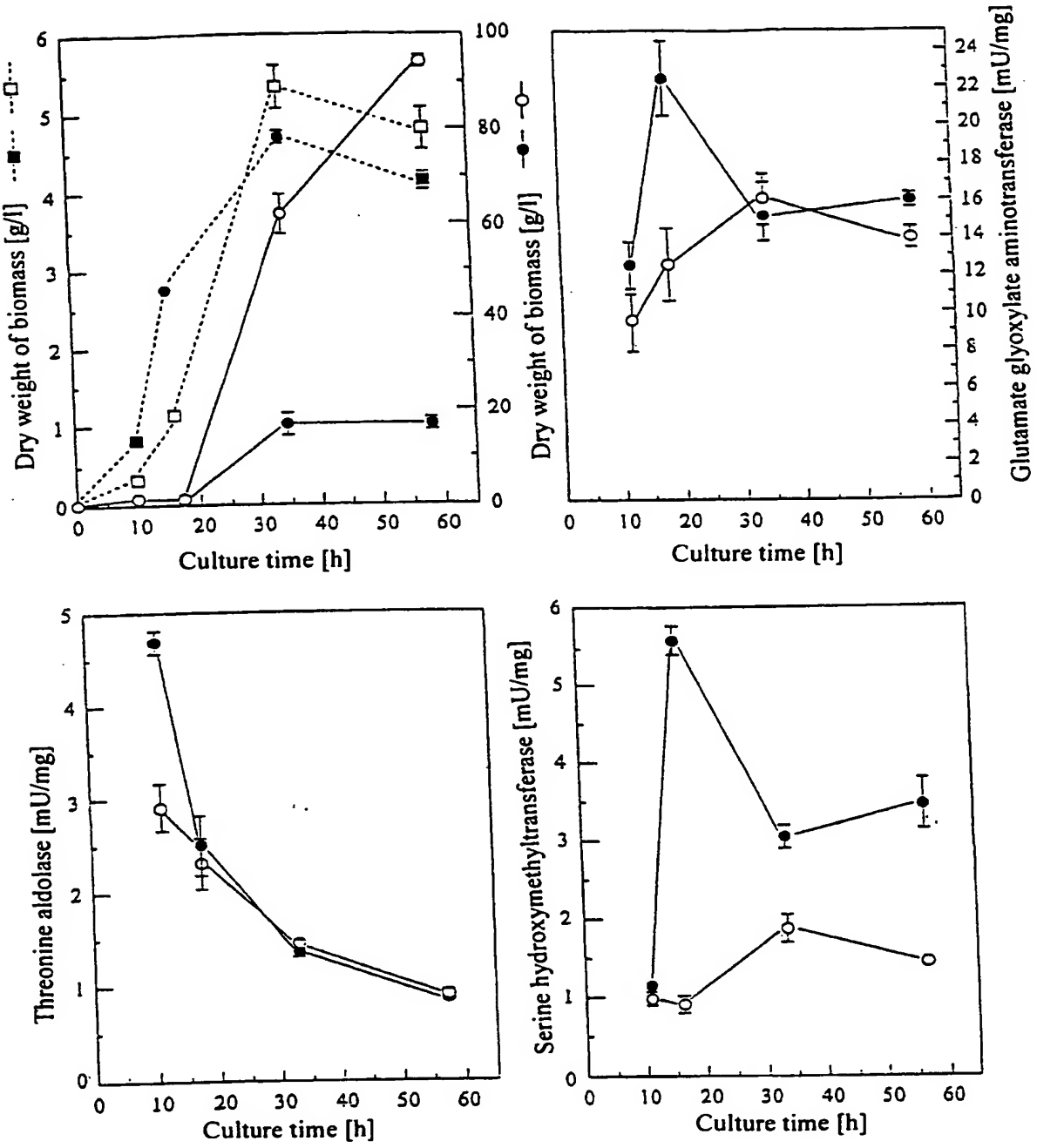

Dry weight of biomass [g/l]
Culture time [h]
Glutamate glyoxylate aminotransferase [mU/mg]
Threonine aldolase [mU/mg]
Serine hydroxymethyltransferase [mU/mg]

Fig. 7

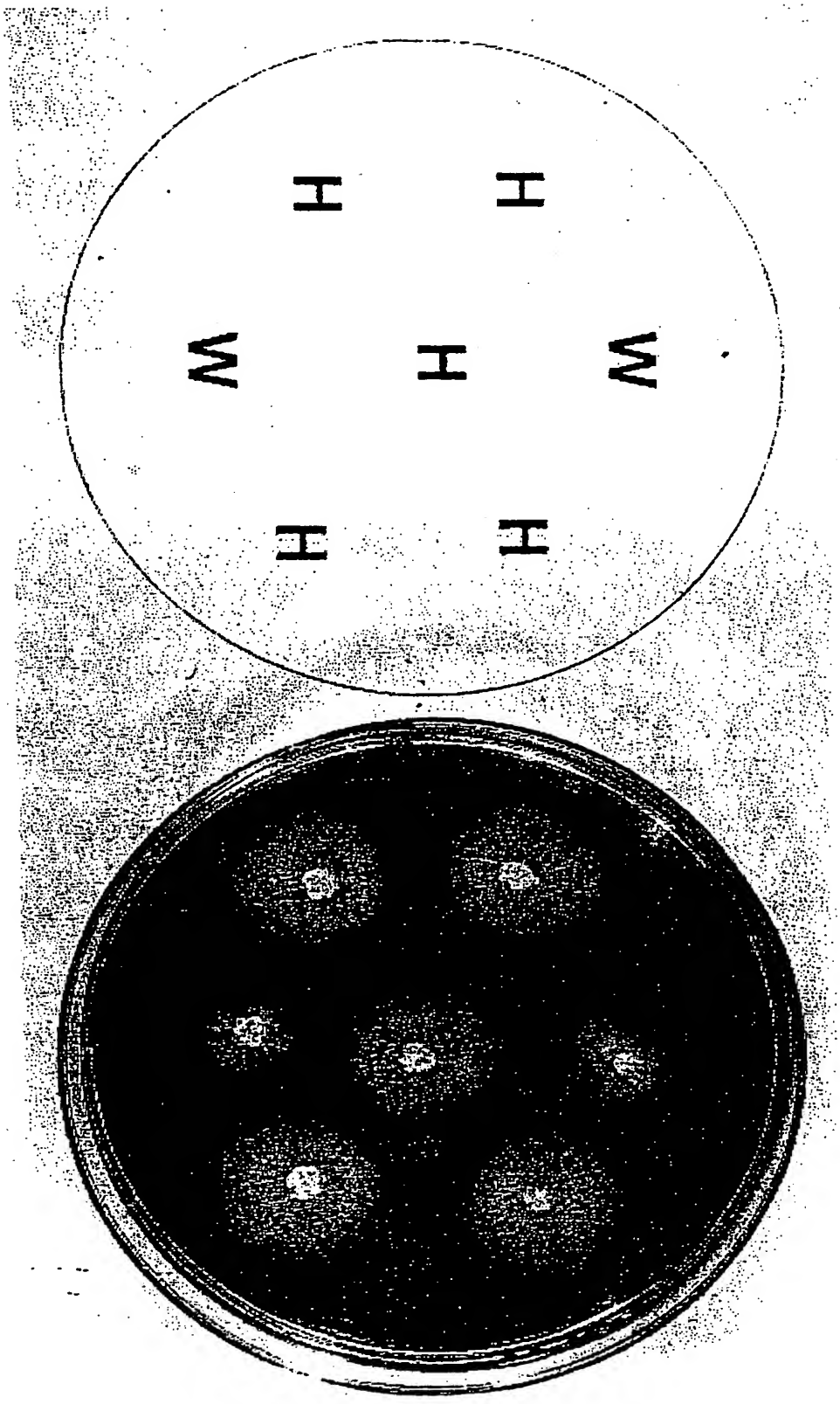
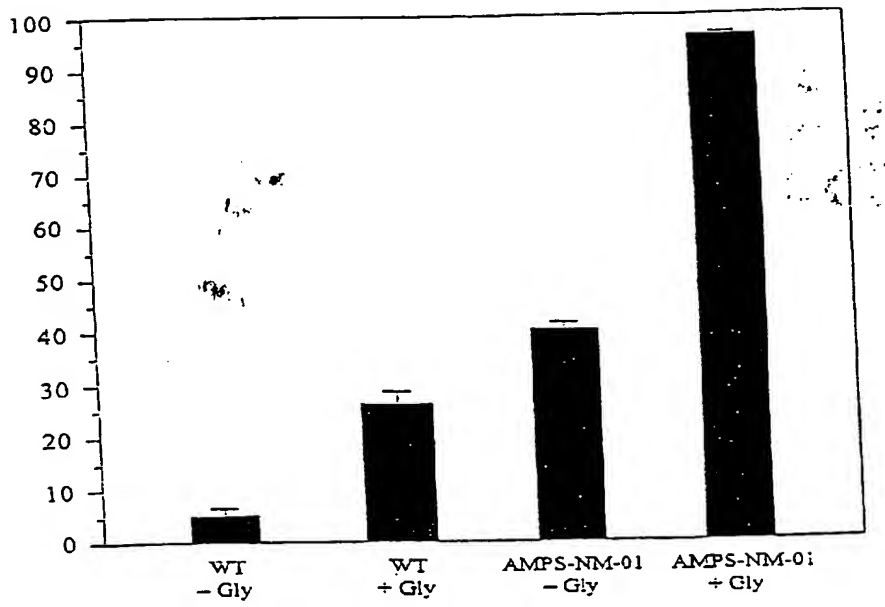


Fig. 8

Drawings


100
90
80
70
60
50
40
30
20
10
0
WT
– Gly
WT
+ Gly
AMPS-NM-01
– Gly
AMPS-NM-01
+ Gly

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